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An LC–MS/MS assay to determine plasma pharmacokinetics of cyclic thymic hexapeptide (cTP6) in rhesus monkeys

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ABSTRACT

A robust and simple method for absolute quantification of a novel bidirectional immunomodulatory drug candidate, cyclic thymic hexapeptide (cTP6), in rhesus monkey plasma was developed and validated by liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS). Plasma proteins were precipitated by adding four volumes of acetonitrile. Peptides in the supernatant were separated by liquid chromatography on an Agilent Zorbax Eclipse Plus-C18 chromatographic column with gradient elution using 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in methanol (mobile phase B) at 0.2 mL/min. The analytes were identified by triple quadrupole mass spectrometry in positive ion-mode. The assay was linear over a concentration range of 10–5000 ng/mL for cTP6, with a lower limit of quantification (LLOQ) of 10 ng/mL. Intra- and inter-day precision of the assay at three concentrations were 1.51–7.70% with accuracy of 95.1–104.2%. The average recovery of cTP6 for three concentration levels was 59.6–64.0%. No significant matrix effect was observed. Peptide cTP6 was detected in plasma of live rhesus monkeys up to 6–8 h after intra-muscular injection. The half-life was 2.24–2.95 h. The result revealed a nonlinear pharmacokinetic response to increasing doses of cTP6 (100, 200, 500 μg/kg). For the multiple dose study of cTP6, the drug did not accumulate during daily administration at 100 μg/kg for 7 consecutive days in rhesus monkeys.

1. Introduction

Cyclic thymic hexapeptide (cTP6) is a novel synthetic analog of thymopentin (TP5). Thymopentin (TP5) is a bidirectional immunomodulatory pentapeptide drug used clinically for treatment of chronic hepatitis B, primary and secondary T cell deficiency, auto-immune diseases, T cell immune hypofunction, AIDS, SARS, and as an adjunct in anti-tumor therapy. The amino acid sequence of TP5 (Arg-Lys-Asp-Val-Tyr) corresponds to the active site (32–36) of the natural hormone thymopoietin (TMPO) [1], which is known as one of the most active thymic hormones from bovine thymus. However, this linear pentapeptide has been reported to be very sensitive to protein enzymes (such as aminopeptidases), and the plasma half-life of TP5 is extremely short (30 s in human plasma) [2]. In order to optimize the pharmacokinetic characteristics *in vivo*, the novel cyclic hexapeptide cTP6 was designed and synthesized based on the linear structure of TP5. Cyclic thymic hexapeptide is homologous to TP5 but with the addition of a cysteine that links to the terminal tyrosine by an amide bond to form the cyclic (Cys-Arg-Lys-Asp-Val-Tyr-). Fig. 1 illustrates the structures of TP5 and cTP6 and their amino acid sequences. Preliminary studies *in vitro* demonstrated that cTP6 had an enhanced effect on immune cells compared to TP5 [3,4]. In the further development of the candidate drug cTP6, quantitation of *in vivo* cTP6 levels is important for evaluating pharmacokinetics and to determine the dose-effect and dose-toxicity relationship. Hence, there is a great need for a reliable, accurate, and sensitive bioanalytical assay for cTP6.

Wang et al. used fluorescence spectrophotometry to investigate the pharmacokinetics of fluorescein isothiocyanate (FITC)-labeled TP5 [5], but the linearity range of 0.05–2.0 μg/mL was not suitable to the present cTP6 study. Liquid chromatography tandem mass spectrometry (LC–MS/MS) is a powerful tool for both qualitative and quantitative analysis of peptide-derived drugs in biological

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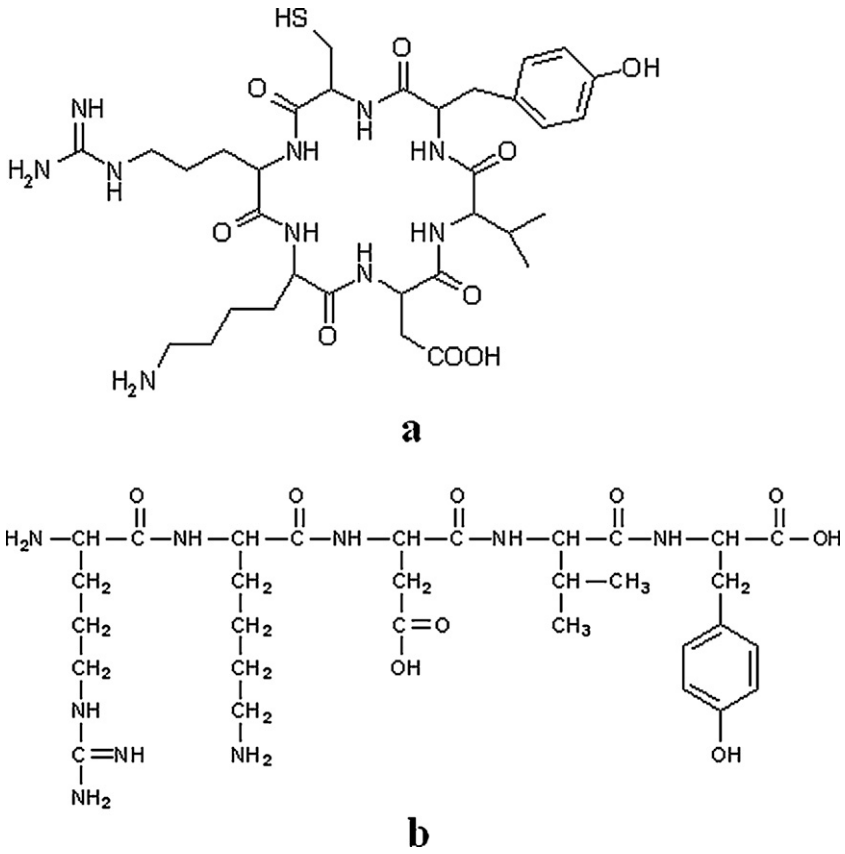

Fig. 1. Structures of (a) cyclic thymic hexapeptide and (b) thymopentin (used as the internal standard).

matrices with high selectivity, sensitivity, and throughput [6–10]. Mock et al. [11] described a sensitive LC–MS/MS method for the quantification of a synthetic cyclic heptapeptide analog of α-melanocyte-stimulating-hormone. A low quantitation limit of 5 ng/mL was achieved in rat plasma. Similarly, Hatziieremia et al. [12] developed an LC–MS/MS method for the quantification of the cyclic heptapeptide melanotan-II (MT-II), a synthetic analog of the natural melanocortin peptide, in mouse plasma and brain homogenate. Recently, a fast and sensitive method for the quantification of an artificially synthesized peptide-derived bombesin/gastrin releasing peptide antagonist, RC-3095, in human plasma was reported using API 4000 mass spectrometer [13]. Indeed, LC–MS/MS has become the preferred analytical technique for quantification of relatively large peptides. Two large peptide-derived drugs, enfuvirtide (MW 4492 Da) and tifuvirtide (MW 5037 Da), and one enfuvirtide metabolite, were quantified in human plasma using LC–MS/MS [14]. The linear range of this method was 20–10,000 ng/mL for the two drugs and 20–5000 ng/mL for the metabolite.

We describe a simple and sensitive quantitative method using precipitation of plasma proteins followed by LC–MS/MS analysis to measure cTP6 down to 10 ng/mL in small plasma volumes (50 μL). TP5 was used as internal standard (IS). A triple quadrupole mass spectrometer operating in positive electrospray ionization mode with multiple reaction monitoring (MRM) was used to detect cTP6 and IS transitions of m/z 766.0 → 136.4 and m/z 680.6 → 400.2. This assay has a wide linear range (10–5000 ng/mL) with intra- and inter-day variation less than 8% and recovery of about 60%. The method was applied successfully to the determination of pharmacokinetic parameters of cTP6 in rhesus monkeys following single intra-muscular injections of three different doses (100, 200, or 500 μg/kg) or daily injections of one dose (100 μg/kg) for 7 days. This is the first report of an LC–MS/MS method capable of determining the novel candidate peptide drug cTP6 for preclinical and future clinical studies.

2. Experimental

2.1. Reagents and chemicals

The cyclic thymic hexapeptide and the internal standard thymopentin were supplied by Baolijian Corporation (Dongguan, Guangdong province, PR China) at purities above 99.0%. Methanol and acetonitrile of HPLC grade were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid (FA, 96%) was purchased from Tedia Company (USA). Deionized water was supplied by the Institute of Microbiology and Epidemiology and was filtered through a 0.2 μm microporous membrane before use. Other chemicals were all of analytical grade.

2.2. LC–MS/MS analysis

High performance liquid chromatography was performed using an Agilent 1100 system (Wilmington, DE, USA) consisting of a vacuum degasser, a binary pump, and an autosampler. The HPLC system was coupled to an API 4000 triple-quadrupole mass spectrometer with a turbo spray interface (Applied Biosystems, Foster City, CA, USA). Chromatographic separation was carried out using an Agilent Zorbax Eclipse Plus-C18 column (50 mm × 2.1 mm i.d. particle size 5 μm). All the experiments were carried out on the LC–MS/MS system. Data acquisition and processing employed Analyst 1.4.2 software (Applied Biosystems, Foster City, CA, USA).

The chromatographic separation was performed with a linear gradient elution at a flow rate of 0.2 mL/min. Mobile phase A was 0.1% (v/v) formic acid in water, and mobile phase B was 0.1% (v/v)

formic acid in methanol. The gradient started at 10% of mobile phase B and linearly rose to 90% B over 1.8 min. Subsequently, the eluent composition was maintained for 2 min before it was decreased to 10% mobile phase B for re-equilibration. The total run time was 14 min.

The mass spectrometer was operated in positive ion mode after passing a mass calibration and sensitivity test using polypropylene glycol (PPG) standard (supplied by Applied Biosystems Company, Foster City, CA, USA) according to the user manual. The two analytes cTP6 and internal standard TP5 were simultaneously monitored by multiple reaction monitoring (MRM) mode. Quantitation was performed using the specific transition (SRM, single reaction monitoring) m/z 766.0 → 136.4 for cTP6 and m/z 680.6 → 400.2 for TP5, respectively. The ion source parameters were optimized and set as follows: collision gas (CAD), 10 psi; curtain gas (CUR), 10 psi; gas1, 30 psi; gas2, 60 psi; ion spray voltage, 5500 V; temperature, 700 °C; declustering potential (DP), 145 V for cTP6 and 137 V for TP5; collision energy (CE), 60 eV for cTP6 and 44 eV for IS; entrance potential (EP), 5 V; collision cell exit potential (CXP), 10 V.

2.3. Preparation of calibration curve and quality control

A stock solution of cTP6 was prepared by dissolving the solid powder into methanol (at about 1 mg/mL). Successive dilutions from this stock solution with water gave working solutions at concentrations of 20, 50, 100, 200, 500, 1000, 2000, 5000, and 10,000 ng/mL. Thymopentin was dissolved in water (at about 1 mg/mL), then freshly diluted to 500 ng/mL before use.

Calibration curves and quality control samples were prepared by spiking deactivated blank monkey plasma with equal volume of the above-mentioned working solutions. The final concentrations of cTP6 in calibration curves were 10, 25, 50, 100, 250, 500, 1000, 2500, and 5000 ng/mL and quality control samples were 50, 500, and 5000 ng/mL. Each sample contained the same amount (500 ng/mL) of the IS. All calibration samples and QC samples were prepared daily to avoid potential degradation or adsorption problems.

2.4. Sample preparation

Blank plasma was obtained from the collected plasma of untreated rhesus monkeys. All the plasma samples and blank plasma were firstly deactivated by adding EDTA (pH 4.5, 50 μL of plasma added to 20 μL of 50 mg/mL EDTA) before use. After each aliquot of plasma sample was deactivated, 50 μL of study sample, calibration standard, or QC sample was deproteinized by protein precipitation. Briefly, an aliquot of 20 μL IS was introduced to 50 μL plasma samples and mixed gently. Then, 0.2 mL acetonitrile (ACN) was added into each mixture followed by vortexing for 3 min. Samples were then centrifuged at 14,000 rpm for 5 min at 4 °C. The upper layer was transferred into a new tube and evaporated to dryness under a gentle stream of nitrogen. The dried residues were reconstituted with 50 μL aqueous solution containing 5% (v/v) methanol and 0.1% (v/v) formic acid (FA). An aliquot of 10 μL of each sample was injected into the LC–MS/MS system for analysis.

2.5. Method validation

The validation parameters of the quantitative method to determine cTP6 in monkey plasma were evaluated according to US Food and Drug Administration (FDA) bioanalytical method validation guidance [15].

The selectivity of the method was evaluated by analysis of six blank plasma samples from six different untreated-monkeys for interference at the retention times of the analyte and IS. The lower limit of quantification (LLOQ) in this experiment was determined by finding the lowest concentrations with a signal to noise > 5 over six independent runs with CV% accuracy within 20% and precision within 20% for every run. The intra- and inter-day precision and accuracy were evaluated by parallel analytical runs performed on the same day or on four consecutive days. Each analytical run consisted of a matrix blank, a set of calibration standards (see Section 2.3), six replicate LLOQ samples, and a set of low, medium, and high concentration QC samples. The accuracy of the assay was determined by comparing the nominal concentrations with the corresponding calculated concentrations. The acceptance criteria were precision within 15% and accuracy between 85 and 115% of the nominal value. The recovery of the analyte cTP6 was investigated by comparing peak areas of extracted QC plasma samples (c) with those of post-extraction blank plasma spiked at corresponding concentrations (b). The matrix effect was determined by comparing the peak areas of post-extraction blank plasma spiked at concentrations of QC samples (b) with the areas obtained by direct injection of corresponding standard solutions (a). Recovery and matrix effect were calculated using Eqs. (1) and (2). Process efficiency was used to optimize plasma sample preparation method. Process efficiency was calculated using Eq. (3).

$$\text{Recovery} = \frac{\text{area } c}{\text{area } b} \quad (1)$$

$$\text{Matrix effect} = \frac{\text{area } b}{\text{area } a} \quad (2)$$

$$\text{Process efficiency} = \frac{\text{area } c}{\text{area } a} \quad (3)$$

Stability tests were performed for analyte-spiked plasma samples under various conditions including three freeze–thaw cycles, 60 days storage at −70 °C, and storage at room temperature for 8 h, by analyzing six replicates at low, medium, and high QC concentrations.

2.6. Data analysis

All chromatograms were processed using the automatic integration software module in Analyst 1.4.2 followed by manual check to confirm proper integration of each peak area. Here the peak area ratios of cTP6 to IS (TP5) versus corresponding concentrations were used for the linear least-squares regression of the calibration lines and for determination of slopes (A), intercepts (B), and correlation coefficients. Unknown sample concentrations of cTP6 were calculated from the linear regression equation (4) with a weighted factor of $1/x$.

$$Y = AX + B \quad (4)$$

2.7. Pharmacokinetics studies

Rhesus monkeys were supplied by the Animal Raising Center of Academy of Military Medical Sciences. The animals were housed in a temperature (25 ± 1 °C), and humidity (55 ± 5%) controlled room under a 12 h light–dark cycle, and fed a standard diet. All the experiments involving animals were approved by Institutional Ethical Committee for Care and Use of Laboratory Animal of Academy of Military Medical Sciences.

In single dose studies, nine normal rhesus monkeys of about 4 kg were randomly divided into 3 groups and administered single doses of cTP6 of 100, 200, or 500 μg/kg. The powder of cyclic hexapeptide was dissolved in physiologic saline to be 1, 2.5, and 5 mg/mL. In the each dose group, 0.34–0.41 mL of saline of cTP6 were injected into monkeys according each body weight. After intramuscular injection of the saline solution with cTP6, 1 mL of blood was drawn from the femoral vein of each rhesus monkey at 0, 5, 10, 15, 20, 30, 45 min, 1, 2, 3, 4, 6, and 8 h after dosing.

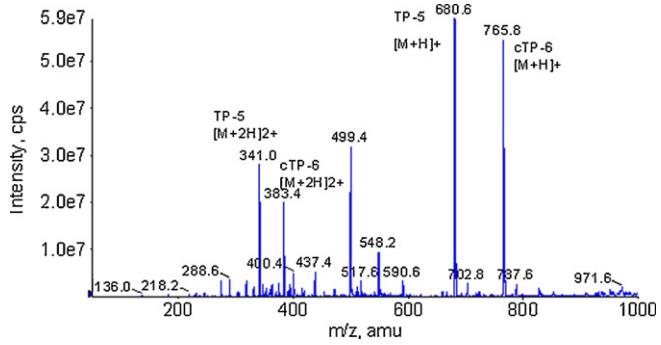

Fig. 2. Q1 full-scan mass spectrum of cTP6 and TP5 (IS) mixture (5 μg/mL) obtained by positive ESI ionization using direct syringe infusion at a flow rate of 10 μL/min.

In multiple dose studies, the low dose group in single dose studies was administered 100 μg/kg once daily for 7 consecutive days. Blood samples were drawn in the same manner as the single-dose groups on days 1 and 7. But on days 2–6, blood samples were collected just prior to administration and at 30 min after administration.

All blood samples were drawn directly into heparinized vacuum blood collection tubes and immediately centrifuged at 5000 × *g* for 5 min at 4 °C to harvest plasma. Plasma samples were then kept at −70 °C before analysis.

3. Results and discussion

Absolute quantification of peptide-derived drugs in biological matrices is still a challenge. The difficulties include poor sensitivity (due to poor ionization efficiency in mass spectroscopy), high interference from biological matrices (due to the high similarity with endogenous substance in biological matrix), and instability of target analyte in biological matrices (due to sensitivity to enzymes in biological matrices). Therefore, emphasis was placed on optimization of sample preparation and LC–MS/MS parameters as these are the most important factors affecting sensitivity, recovery, stability, and efficiency (analysis time).

3.1. MS optimization

Optimization of MS first involved tuning of MS parameters in positive and negative ionization modes for cTP6 and TP5 (IS) using a 5000 ng/mL tuning solution (methanol:H_2O at 1:1). Both peptides responded best to positive ionization. Fig. 2 depicts the Q1 full-scan mass spectrum of a 5000 ng/mL mixture solution of cTP6 and TP5 (IS) in positive mode. Protonated molecular ions $[M+H]^+$ at *m*/*z* 765.8 and 680.6 were present as major peaks for cTP6 and TP5 (IS), respectively. Doubly charged ions were also observed at *m*/*z* 383.4 for cTP6 and 341.0 for TP5. The identifications matched the theoretical molecular weights of cTP6 (765.0 Da) and TP5 (679.7 Da).

Following the optimization of ions 765.8 and 680.6, they were used as the precursor ions to obtain product ion spectra of cTP6 and TP5 (IS). Tandem spectrums of cTP6 and TP5 are shown in Fig. 3a and b. The two parameters collision energy (CE) and declustering potential (DP) were carefully optimized to produce the maximum response for the two peptides. The cyclic chemical structure of cTP6 is more stable than the linear structure of TP5, so a higher energy was needed to fragment cTP6. In Fig. 3a, a precursor ion of cTP6 at *m*/*z* 766.0 gave major product ions at *m*/*z* 136.4, *m*/*z* 129.2, and *m*/*z* 101.0, suggesting the immonium ions of tyrosine, arginine, and lysine, respectively. The transition of 766.0 → 136.4 was chosen for the quantification studies since the SRM transition exhibited the highest sensitivity. For TP5 (Fig. 3b), the parent ion at 680.6 was fragmented into serial product ions (b1, b2, b3, a1 ions etc.). The transition 680.6 → 400.2 was only employed for quantification. Another interesting phenomenon (not related to our objective) was noticed in the MS/MS spectrums of TP5. Several ions with strong signals always presented with their accompanying neutral fragments with loss of ammonia, such as a1, b1, b2, and b3 in MS/MS spectrum of TP5. Moreover, a molecular ion of TP5 was also observed with loss of ammonia. The cause of this phenomenon is not clear and is still under investigation.

3.2. LC optimization

The optimal chromatographic conditions were obtained by evaluating the different mobile phase, elution type, flow rate, as well as type of chromatographic column. Acetonitrile and methanol were tested in the organic mobile phase. It was found that peptides gave similar responses and resolution in the two types of mobile phase, but a methanol-based mobile phase had lower background noise. The inclusion of 0.1% FA greatly enhanced the intensities of the peaks. It was also noted that gradient elution dramatically improved response intensity, resolution, and peak shape. The initial percent of organic phase was always lower than 20% at the start of gradient elution. Out of a number of commercially available C18 columns evaluated, the Zorbax Eclipse Plus-C18 column was found to give the most satisfactory chromatography, and the 5 cm column length helped to reduce the whole analysis time.

3.3. Sample preparation

Protein precipitation and solid phase extraction (SPE) are commonly used as sample clean-up methods for peptide-derived drugs prior to LC–MS analysis [16]. A simple protein precipitation (PPT) method was selected for simplicity and low cost. Process efficiency is determined by the combination of matrix effects and recovery of the analyte during the sample preparation process (Section 2.5). The optimal conditions for extraction of analyte can be obtained by evaluating the value and stability of process efficiency of cTP6 to that of the IS analysis of the experimental results. The biggest and most stable process efficiency was used as criterion for the selection of the optimal extraction conditions. Two factors of extraction were considered and optimized, extraction agent (methanol, acetonitrile, and acetone) and the volume ratio of plasma to agent (1:2, 1:4, 1:8, 1:10, 1:20). Methanol and acetone were less than half as efficient as acetonitrile, so acetonitrile was selected. The values of process efficiency decreased when the ratio increased from 1:2 to 1:20. The most stable process efficiency was obtained when used at a ratio of 1:4 (S.D. < 20%). The results indicated that the volume ratio of 1:4 plasma to acetonitrile yielded the highest extraction efficiency and stability.

3.4. Strategies to resolve instability of analytes in plasma sample

Degradation of both the analyte cTP6 and the IS TP5 in plasma was observed and measured. Several kinds of degradation were observed. Loss of N-terminal arginine or C-terminal tyrosine was most common, particularly in the presence of aminopeptidase and carboxypeptidase. Previous reports have noted enzymatic degradation of thymopentin [2,17]. The chelator EDTA was reported to bind zinc ions at the active site of metallo-proteases such as carboxypeptidase A and B and arninopeptidase N [17], so 20 μL of 50 mg/mL aqueous EDTA (pH 4.5) was added to inhibit the activity of these enzymes in plasma. This deactivation method increased process efficiency to approximately 60%.

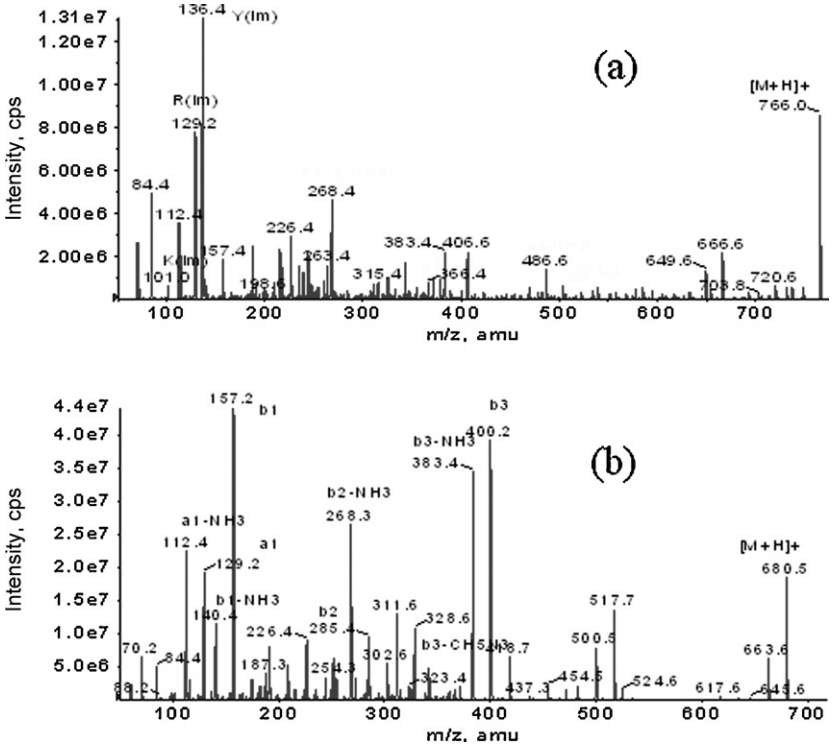

Fig. 3. Product ion scans of (a) cTP6 (precursor ion *m*/*z* 766.0) and (b) TP5 (precursor ion *m*/*z* 680.6).

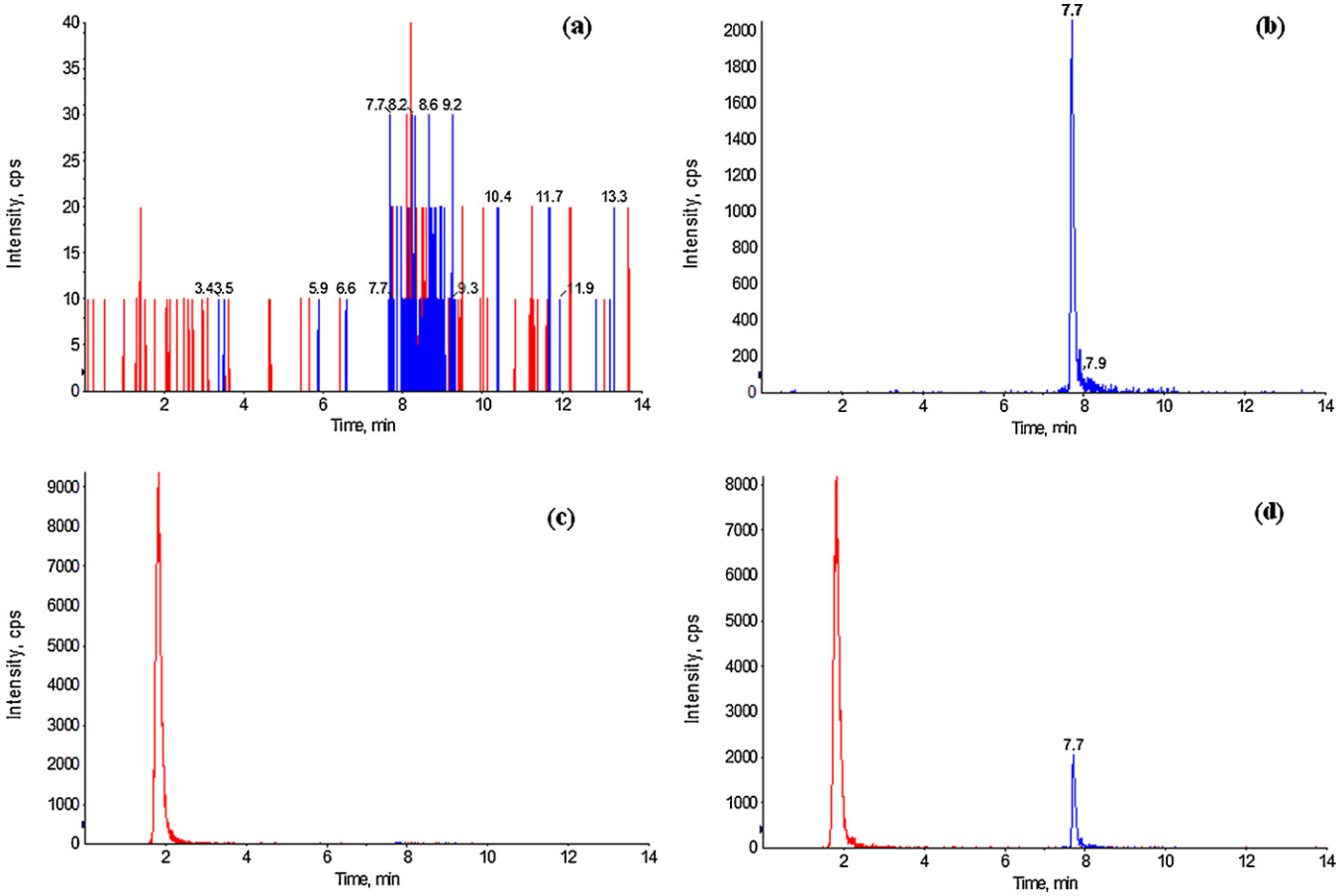

Fig. 4. Representative LC-MRM chromatograms for analytes: (a) blank plasma; (b) blank plasma spiked with 500 ng/mL cTP6; (c) blank plasma spiked with 500 ng/mL IS; (d) extract of plasma sample from No. 8 rhesus monkey at 15 min after intramuscular injection of 500 μg/kg cTP6.

Table 1
Accuracy and precision of standards in pooled plasma from normal monkeys at three concentration levels.

	50 ng/mL	500 ng/mL	5000 ng/mL
Intra-day (n = 5)			
Mean (ng/mL)	51.95	497	5207.5
Accuracy (%)	103.9	99.4	104.2
C.V.%	4.40	6.15	1.51
R.E.%	3.90	−0.60	4.15
Inter-day (n = 15)			
Mean (ng/mL)	48.6	521.2	4756
Accuracy (%)	97.2	104.2	95.1
C.V.%	7.70	2.52	4.42
R.E.%	−2.8	4.24	−4.88

Accuracy = measured concentration/nominal concentration × 100%; C.V.% (coefficient of variation) = S.D./mean × 100%; R.E% (percent relative error) = [(mean/nominal) − 1] × 100%.

Table 2
Recovery and matrix effect of three concentrations of cTP6.

Name	Concentration (ng/mL)	Mean ± S.D.	
		Matrix effect (%)	Recovery (%)
cTP6	50	95.5 ± 4.81	64.0 ± 2.61
	500	98.4 ± 2.60	59.6 ± 2.38
IS	5000	102 ± 3.57	61.0 ± 0.75
	500	98.5 ± 4.26	62.7 ± 1.50

3.5. Method validation

3.5.1. Selectivity

The selectivity of the method was evaluated by analysis of six blank plasma samples from six different untreated-monkeys for interference at the retention times of the analyte and IS. Representative LC-MRM chromatograms for blank plasma samples (a), blank plasma spiked with cTP6 (b), blank plasma spiked with IS (c), and plasma sample of treated rhesus monkey (d) are shown in Fig. 4. The retention times of cTP6 and IS are 7.7 min and 1.7 min respectively. No signal similar to cTP6 (7.7 min) or internal standard (1.7 min) was found in the chromatogram of blank plasma, which suggested that there were no endogenous substances that could interfere with the analysis of cTP6 and IS (thymopentin) in blank plasma.

3.5.2. Sensitivity and linearity

The assay was linear over the concentration range of 10–5000 ng/mL. The correlation coefficients for the calibration curves (weighted by 1/x) ranged from 0.9966 to 0.9993. The calibration curves were defined by slopes of 0.00033 (±0.00015) mL/ng and intercepts of −0.0055 (±0.0060) for cTP6. A typical linear regression equation for the calibration curve was list below.

$$y = 0.000176x + 0.00183 \quad (r = 0.9990) \tag{5}$$

where "y" represents the peak area ratios of cTP6 to that of IS, and "x" represents the plasma concentration of cTP6 (ng/mL). The lower limit of quantification (LLOQ) in this experiment was found to be 10 ng/mL with a S/N ratio above 10 over six runs. Deviations of LLOQ from the nominal concentrations over six runs were less than ±13.8% and the accuracy were within ±5.5%. The upper limit of quantification (ULOQ) in this study was defined as 5000 ng/mL using the same criteria.

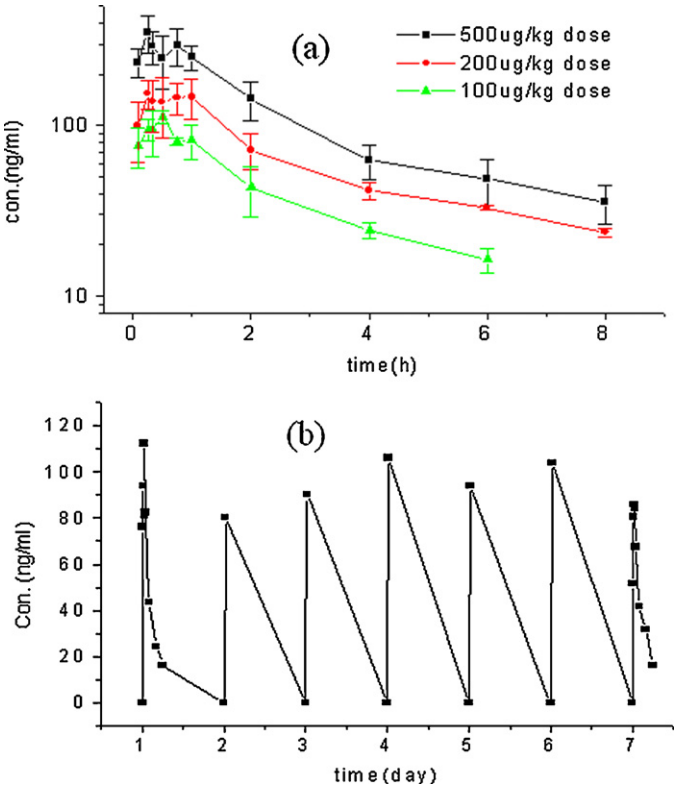

Fig. 5. Concentration–time profiles of cTP6 following (a) a single dosage (100, 200 or 500 μg/kg). Data are mean ± S.D., n = 3 per group) and (b) daily intramuscular injection of 100 μg/kg for 7 consecutive days in monkeys (n = 3).

Table 3
The stability of cTP6 in plasma under various storage conditions (post preparation and storage at room temperature for 8 h; three freeze–thaw cycles; or storage 60 days at −70 °C.

Concentration (ng/mL)	Post preparation (8 h)		Freeze–thaw		Long-term (60days)	
	Mean ± S.D.	Accuracy	Mean ± S.D.	Accuracy	Mean ± S.D.	Accuracy
50	49.84 ± 4.91	99.68	48.76 ± 6.00	99.04	49.72 ± 5.77	99.44
500	495.2 ± 4.30	99.04	518.6 ± 3.13	103.6	503.6 ± 2.18	100.7
5000	5144 ± 3.97	102.9	4776 ± 3.17	95.52	5236 ± 4.47	104.7

Accuracy expressed as measured concentration/nominal concentration × 100%.

Table 4
Pharmacokinetic parameters for 9 rhesus monkeys after the administration of a single dose of cTP6 (n = 3 per group).

Dose (μg/kg)	$AUC_{0-\infty}$ (ng h/mL)	MRT (h)	CL (L kg^{-1} h^{-1})	$T_{1/2}$ (h)	K_{el}	C_{max} (ng/mL)	T_{max} (h)
100	311 ± 47	1.98 ± 0.07	0.326 ± 0.05	2.24 ± 0.423	0.315 ± 0.03	112 ± 9.81	0.50 ± 0
200	587 ± 37	2.63 ± 0.19	0.341 ± 0.02	2.95 ± 0.157	0.235 ± 0.01	159 ± 27.4	0.69 ± 0.4
500	999 ± 202	2.37 ± 0.08	0.51 ± 0.10	2.56 ± 0.27	0.27 ± 0.03	352 ± 87.4	0.25 ± 0

Table 5
Day 1 vs. day 7 pharmacokinetic parameter of cTP6 in rhesus monkeys following daily administration (100 μg/kg) for 7 consecutive days ($n = 3$).

Parameters	1st day	7th day	P
AUC_{0-6} (ng h/mL)	258.7 ± 44.8	248.6 ± 48.5	0.770
$AUC_{0-\infty}$ (ng h/mL)	311.3 ± 47.3	288.9 ± 40.4	0.567
MRT (h)	1.977 ± 0.07	2.162 ± 0.107	0.070
CL (L kg^{-1} h^{-1})	0.326 ± 0.05	0.351 ± 0.048	0.553
V_{ss} (L kg^{-1})	0.645 ± 0.10	0.761 ± 0.138	0.297
$T_{1/2}$ (h)	2.209 ± 0.18	2.243 ± 0.423	0.906
K_{el}	0.315 ± 0.03	0.317 ± 0.061	0.968
C_{max} (ng/mL)	112.3 ± 9.81	100.8 ± 24.4	0.490
T_{max} (h)	0.50 ± 0	0.527 ± 0.211	0.838
$AR_{C_{max}}$	0.90		
AR_{AUC}	0.91		

3.5.3. Accuracy and precision

Summary intra- and inter-day precision and accuracy results of cTP6 in plasma are presented in Table 1. For intra-day precision, the CV of QC samples was between 1.51 and 6.15% and the accuracy of QC samples was between 99.4 and 104.2%. For inter-day precision, the CV of QC samples was between 2.52 and 7.70% and the accuracy of QC samples was between 95.1 and 104.2%.

3.5.4. Recovery and matrix effect of the method

Results of recovery and matrix effect are presented in Table 2. The mean matrix effect of cTP6 ranged from 95.5 ± 4.81% to 102 ± 3.57% at concentrations of 50, 500, and 5000 ng/mL and the matrix effect of IS was 98.5 ± 4.26%. As the value of matrix effect was about 100%, there was no significant matrix enhancement or matrix suppression in monkey plasma at three different concentration levels. The mean recoveries of cTP6 were 64.0 ± 2.61%, 59.6 ± 2.38%, and 61.0 ± 0.75% at concentrations of 50, 500, and 5000 ng/mL, respectively. Similar recoveries of internal standard were obtained.

3.5.5. Robustness of the method

The measured concentrations obtained from all stability conditions were compared with the nominal concentrations of three QC samples (Table 3). The auto sampler stability was investigated after exposure of the post-extraction plasma samples to room temperature on the autosampler for 8 h. The result showed accuracies were between 99.04 and 102.9%, and standard deviations were all less than 4.91%. Therefore, post-preparation and reconstituted cTP6 was stable when left in the autosampler for 8 h. After three freeze–thaw cycles, the accuracies were between 95.52 and 103.6%, and standard deviations were less than 6.00%. After 60 days storage, the accuracies were between 99.44 and 104.7% and standard deviations were less than 5.77%. The results of three freeze–thaw cycles and long-term stability study showed that cTP6 was stable after sample preparation.

3.6. Application in rhesus monkeys

3.6.1. Single dose studies

Following a single intramuscular administration, a peak plasma concentration (C_{max}) was observed between 0.25 and 0.69 h for all three doses. The plasma terminal elimination $T_{1/2}$ varied from 2.24 h to 2.95 h in the three dosage groups. $AUC_{0-\infty}$ values of the three doses were estimated to be 311 ± 47, 587 ± 37, and 999 ± 202 ng h/mL, respectively. The C_{max} values were 112 ± 9.81, 159 ± 27.4 and 352 ± 87.4 ng/mL, respectively. Fig. 5a shows the mean plots of plasma concentration-time after administration of cTP6 to 9 rhesus monkeys at the three different dosages. The pharmacokinetic parameters of cTP6 are summarized in Table 4. Across the investigated dosage range in monkeys (100, 200, and 500 μg/kg), C_{max} ratios (1:1.4:3.1) and $AUC_{0-\infty}$ ratios (1:1.9:3.2) increased as the dose increased (1:2:5) but not proportionally. The results supported non-linear rather than linear plasma pharmacokinetics of cTP6.

3.6.2. Multiple-dose studies

The mean plasma concentration–time profile of cTP6 on days 1–7, following a daily injection at 100 μg/kg for 7 consecutive days is displayed in Fig. 5b. The analysis of variance of the plasma concentration between days 1 and 7 showed no difference ($P > 0.5$, data not shown). The observed AUC_{0-6} and C_{max} of cTP6 for the first dose on day 1 was 258.7 ± 44.8 ng h/mL and 112.3 ± 9.81 ng/mL, respectively. For the last dose on day 7, the AUC_{0-6} and C_{max} were 248.6 ± 48.5 ng h/mL and 100.8 ± 24.4 ng/mL, respectively. The accumulation ratios (ARs) of the AUC and C_{max} for cTP6 were 0.91 and 0.90, based on the day 7 to day 1 ratios of C_{max} and AUC_{0-6}. The pharmacokinetics comparison of days 1 and 7 are shown in Table 5. The analysis of variance of the pharmacokinetic parameters between day 1 and day 7 showed no difference ($P > 0.05$). These results indicate that there was no accumulation during multiple doses at 100 μg/kg in monkeys.

4. Conclusions

A sensitive and simple quantification method based on LC–MS/MS to determine a novel peptide drug candidate, cyclic thymic hexapeptide, in plasma was developed and validated. This is the first method capable of determining cTP6 concentrations in plasma samples after injection in monkeys. A simple protein precipitation procedure was used before samples were injected into the autosampler of the LC–MS/MS system. The method showed good precision, accuracy, high sensitivity (10 ng/mL), low consumption of plasma (50 μL), as well as a wide linear range of 10–5000 ng/mL. Successful analyses of hundreds of plasma samples from rhesus monkeys and rats (data not shown) demonstrated that the method is efficient, reliable, and suitable for preclinical and future clinical studies of cTP6. This is the first study to determinate cTP6 in rhesus monkeys.

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